Copy for the Elected Office (EO/US)

PCT/CA99/01093

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SECHLEY, Konrad, A.
Gowling Lafleur Henderson LLP
Suite 2600
160 Elgin Street
Ottawa, Ontario K1P 1C3
CANADA

Date of mailing (day/month/year)
25 August 2000 (25.08.00)

Applicant's or agent's file reference
08-0881610WO

IMPORTANT NOTIFICATION

International application No.
PCT/CA99/01093

International filing date (day/month/year)
16 November 1999 (16.11.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
IOGEN CORPORATION
400 Hunt Club Road
Ottawa, Ontario K1V 1C1
Canada

State of Nationality: CA
State of Residence: CA
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
NATIONAL RESEARCH COUNCIL OF CANADA
Intellectual Property Office
Building M-58, EG-10
1200 Montreal Road
Ottawa, Ontario K1A 0R6
Canada

State of Nationality: CA
State of Residence: CA
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
The agent's address has also been changeda as mentioned above.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Philippe Bécamel

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

003490288

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 20 June 2000 (20.06.00)	
International application No. PCT/CA99/01093	**Applicant's or agent's file reference** 08-0881610WO
International filing date (day/month/year) 16 November 1999 (16.11.99)	**Priority date** (day/month/year) 16 November 1998 (16.11.98)
Applicant SUNG, Wing, L. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

26 May 2000 (26.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Manu Berrod
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

ENT COOPERATION TRE

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SECHLEY, Konrad A
Gowling, Lafleur Henderson LLP
Suite 2600
160 Elgin Street
Ottawa, Ontario K1P 1C3
CANADA

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	18. 08. 2000
Applicant's or agent's file reference: 08-0881610WO	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01093	16/11/1999	16/11/1998

International Patent Classification (IPC) or both national classification and IPC

C12N15/56

Applicant

IOGEN CORPORATION et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 16/03/2001.

Name and mailing address of the international preliminary examining authority:
European Patent Office - P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk - Pays Bas
Tel. +31 70 340 - 2040 Tx: 31 651 epo nl
Fax: +31 70 340 - 3016

Authorized officer / Examiner
Cupido, M

Formalities officer (incl. extension of time limits)
Sinanovic, E
Telephone No. +31 70 340 2672

I. **Basis of the opinion**

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-38 as originally filed

Claims, No.:

1-28 as originally filed

Drawings, sheets:

1/28-28/28 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. **Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-11,15,16,23-25,27
Inventive step (IS)	Claims	1-28
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

I Documents

The following documents are mentioned in this written opinion; their numbering will be adhered to in the rest of the procedure:

D1: EP 0 828 002 (National Research Council of Canada)
D2: WO 95/29997 (Finnfeeds International)
D3: WO/94/24270 (National Research Council of Canada)
D4: Protein Engineering 7:1379-1386 (Wakarchuk et al., 1994)

II Novelty

1. D1 discloses in example 12 that the chimeric xylanase NI-TX8 keeps 55% of its activity after 60 minutes incubation at 68°C, the activity being measured at 40°C. In example 8 is disclosed that NI-TX-8 has full activity up to pH 6.0. Hence the subject-matter in claims 1, 2, 6, 8-11 lacks novelty in view of Article 33(2)PCT.

2. D2 discloses a method of obtaining a xylanase from *Microtetraspora flexuosa* or *Thermonospora fusca*. These xylanases are after a one minute heat shock at 95°C still active at pH 6.5 and at 40°C and capable to reduce the viscosity of a cereal-based feed. Hence, the subject-matter in claims 23-25 and 27 lacks novelty within the meaning of Article 33(2) PCT.

3. D3 discloses *Bacillus circulans* xylanase mutants having additional disulfide bridges, see table 2 on page 126, resulting in increased thermostability, while retaining their optimal temperature, see table 11 on page 164. Hence the subject-matter in claims 1, 2, 9,10, 15 and 16 lacks novelty as required by Article 33(2) PCT.

4. Whether the thermostable xylanases disclosed in D1-D3 are within the scope of claims 3-5, 7 and 8 cannot be determined. It appears to be unusual to add glycerol or another stabilizer during the pre-incubation step and hence no statement on the novelty of the subject-matter in these claims can be made. The PCT International Preliminary Examination Guidelines III-4.7a state that characterization of a product solely by its parameters should, as a general rule, not be allowed.

5. The subject-matter in claims 12-14,17-22, 26 and 28 appears to be novel.

III Inventive step

1. D1 is regarded as the closest prior art with respect to the question whether the claimed subject-matter involves an inventive step. The underlying technical problem to be solved by the present invention in view of D1 is the provision of further thermostable xylanases that are potentially useful in feed pelleting applications.

2. The solutions provided and claimed by the present invention consist of thermostable xylanases, some comprising a basic amino acid at position 162 or its equivalent. A Q162H xylanase mutant from *T. reesei* has already been disclosed in D1 (mutant NI-TX1). It appears that this mutation has no effect on the thermostability of the xylanase mutant and the presence of this mutation as such, is therefore not regarded to involve an inventive step within the meaning of Article 33(3) PCT.

3. It appears that the increased thermostability of the mutants as claimed in claims is related to the presence of at least one disulfide bridge. D4 discloses that the thermostability of a family 11 xylanase derived from *Bacillus circulans* could be enhanced by the introduction of both intra- and intermolecular disulfide bridges by site-directed mutagenesis. Hence it would be obvious, when trying to solve the above technical problem to introduce at least one disulfide bridge in other family 11 xylanases, with a reasonable chance of success, thereby arriving at the present invention, which hence is regarded to be obvious in the light of the prior art and not to involve an inventive step as required by Article 33(3) PCT.

4. The applications for the preparation of animal feed being poultry or swine feed is also regarded as obvious and not to involve an inventive step.

INTERNATIONAL SEARCH REPORT

Internatio . Application No
PCT/CA 99/01093

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/56 C12N9/24 A23K1/165

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 828 002 A (NATIONAL RESEARCH COUNCIL OF CANADA) 11 March 1998 (1998-03-11) cited in the application page 6, line 10 - line 13; example 12 ---	1,2, 9-11, 23-28 15-22
Y	WAKARCHUK W W ET AL: "THERMOSTABILIZATION OF THE BACILLUS CIRCULANS XYLANASE BY THE INTRODUCTION OF DISULFIDE BONDS" PROTEIN ENGINEERING,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 7, no. 11, 1 January 1994 (1994-01-01), pages 1379-1386, XP002072553 ISSN: 0269-2139 cited in the application the whole document --- -/--	15-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2000	09/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SECHLEY, Konrad A
Gowling, Lafleur Henderson LLP
Suite 2600
160 Elgin Street
Ottawa, Ontario K1P 1C3
CANADA

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	26.02.2001
Applicant's or agent's file reference 08-0881610WO	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01093	16/11/1999	16/11/1998

Applicant
IOGEN CORPORATION et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Sinanovic, E Tel.+31 70 340-2672

Form PCT/IPEA/416 (July 1992)

ENT COOPERATION TRE

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
08-0881610WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/01093	16/11/1999	16/11/1998
International Patent Classification (IPC) or national classification and IPC C12N15/56		
Applicant IOGEN CORPORATION et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
26/05/2000	26.02.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Cupido, M Telephone No. +31 70 340 3374

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA99/01093

I. **Basis of the report**

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-38	as originally filed

Claims, No.:

1-32	as received on	20/11/2000	with letter of	17/11/2000

Drawings, sheets:

1/28-28/28	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-32
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-32
Industrial applicability (IA)	Yes:	Claims	1-32
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

I Documents

The following documents have been taken into consideration:

D1: EP 0 828 002 (National Research Council of Canada)

D2: WO 95/29997 (Finnfeeds Iternational)

D3: WO/94/24270 (National Research Council of Canada)

D4: Protein Engineering 7:1379-1386 (Wakarchuk et al., 1994)

II Novelty

1. D1 discloses the chimeric xylanase NI-TX8 which keeps 55% of its activity after 60 minutes incubation at 68°C, the activity being measured at 40°C. According to figure 7, NI-TX8 exhibits less than 40% activity at at pH 4.5, which is identical to that of the wildtype or natural TrX. Figure 8 of the present application shows that mutants TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 have a much broader pH optimum resulting in about 50% of optimal activity at pH 3.5. However, this pH optimum is also identical to that of the wildtype or natural TrX pH curve. Since the pH optimum curves of the mutants disclosed in D1 and those of the present application are both identical to those of the wildtype or natural TrX, they must also be identical to each other. Apparently, the effects of the pH on xylanase is dependent on the way they have been determined and these effects cannot be used to discriminate the xylanases from the present invention from the isolated xylaneses known from the prior art such as D1. Moreover, the use of the term "about" pH 3.5 (see item VIII below)prevents the invention from being unambiguously distinguished from the prior art.

2. D2 discloses a method of obtaining a xylanase from *Microtetraspora flexuosa* or *Thermonospora fusca*. These xylanases are after a one minute heat shock at 95°C still active at pH 6.5 and at 40°C and capable to reduce the viscosity of a cereal-based feed.

3. D3 discloses *Bacillus circulans* xylanase mutants having additional disulfide bridges, see table 2 on page 126, resulting in increased thermostability, while retaining their optimal temperature, see table 11 on page 164.

4. Whether the thermostable xylanases disclosed in D1-D3 are within the scope of claims 3-5, 7 and 8 cannot be determined. It appears to be unusual to add glycerol or another stabilizer during the pre-incubation step and hence no statement on the novelty of the subject-matter in these claims can be made. The PCT International Preliminary Examination Guidelines III-4.7a state that characterization of a product solely by its parameters should, as a general rule, not be allowed.

5. Hence, the subject-matter in claims 1-32 lacks novelty in view of Article 33(2)PCT.

III Inventive step

1. D1 is the closest prior art with respect to the question whether the claimed subject-matter involves an inventive step. The underlying technical problem to be solved by the present invention in view of D1 is the provision of further thermostable xylanases that are potentially useful in feed pelleting applications.

2. The solutions provided and claimed by the present invention consist of thermostable xylanases, some comprising a basic amino acid at position 162 or its equivalent. A Q162H xylanase mutant from *T. reesei* has already been disclosed in D1 (mutant NI- TX1). It appears that this mutation has no effect on the thermostability of the xylanase mutant and the presence of this mutation as such, is therefore not regarded to involve an inventive step within the meaning of Article 33(3) PCT.

3. It appears that the increased thermostability of the mutants as claimed is related to the presence of at least one disulfide bridge. D4 discloses that the thermostability of a family 11 xylanase derived from *Bacillus circulans* could be enhanced by the introduction of both intra- and intermolecular disulfide bridges by site- directed mutagenesis. Hence it is obvious, when trying to solve the above technical problem to introduce at least one disulfide bridge in other family 11 xylanases, with a reasonable chance of success, thereby arriving at the present invention. The applications for the preparation of animal feed being poultry or swine feed is also regarded as obvious and not to involve an inventive step. The invention is obvious in the light of the prior art and the subject-matter in claims 1-32 do not involve an inventive step as required by Article 33(3) PCT.

Re Item VIII

Certain observations on the international application

1. Claim 1 refers to an isolated, modified family 11 xylanase that exhibits at least 40% of optimal activity from about pH3.5 to about pH 6.0, and from about 40 to about 50°C,said xylanase being thermostable. These properties are not specific features but rather obviously desirable objectives, without indicating the means to achieve said objectives. Claims which attempt to define the invention, or a feature thereof, by a result to be achieved are not allowed, unless the invention can only be defined in such terms and if the result is one which can be directly and positively verified by trial and error, see the PCT preliminary examination Guidelines C-III, 4.7 It is clear in the present case (see for example Table 2 in the description page 19) that the present invention can be described by using technical features. The same objection is made with regard to the method of claim 23, and hence claims 1 and 23 and the claims that are dependent on these claims lack clarity as required by Article 6 PCT.

2. Claim 1 refers to a xylanase that exhibits at least 40% of optimal activity from about pH3.5 to about pH 6.0, and from about 40 to about 50°C. The

meaning of the term "about" is not regarded to be clear in the context of the application as a whole, and prevents the invention from being unambiguously distinguished from the prior art, see the Guidelines C-III, 4.5a.

Increase in catalytic activity and thermostability of the xylanase A of *Streptomyces lividans* 1326 by site-specific mutagenesis

Alain Moreau, François Shareck, Dieter Kluepfel, and Rolf Morosoli

Centre de recherche en microbiologie appliquée, Institut Armand-Frappier, Laval, Québec, Canada

The xylanase A gene from Streptomyces lividans *was modified by site-directed mutagenesis, selecting for mutations that improved the catalytic activity and thermostability of the enzyme. Mutant notation uses the one-letter abbreviation for amino acids. The first and the last letters represent, respectively, the residue to be changed and the replacing residue. The number indicates the position of the substitution. The mutant enzymes F155Y, R156E, R156K, and N173D were respectively 28, 10, 50, and 25% more active than the wild-type enzyme. In addition, the half-lives at 60°C of the R156E and N173D xylanases were respectively 6 and 40 min longer than that of the wild-type enzyme even in the absence of substrate. The favorable single mutations were combined to generate the double mutants E156/173D and K156/173D, which were 22 and 47% less active than the wild type. However, the activity half-life of the E156/173D enzyme at 60°C was twice that of the xylanase A. The pH-activity profiles of all the mutant xylanases were similar to that of the wild-type enzyme.*

Keywords: Xylanase; mutagenesis; thermostable enzyme; *Streptomyces lividans*

Introduction

Xylan is a polysaccharide found in the hemicellulosic fraction of lignocellulose. It is composed of β-1,4-linked D-xylose residues that form the backbone of the polymer chain, which can also carry side-chain substitutions such as α-arabinofuranoside and methylglucuronic and acetic acids. This biopolymer is hydrolyzed by xylanases (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8). The most promising industrial application of xylanases is their use in the bio-bleaching of Kraft paper pulps.[1]

During the last decade, there has been growing public concern over the ecological risks and environmental impacts of toxic compounds generated from the chemical bleaching process used in the pulp and paper industry. The potential of hemicellulases for the bio-bleaching of Kraft pulps has greatly stimulated applied as well as fundamental research in this field. The industrial application of this technology requires highly active and thermostable enzymes. Several thermophilic microorganisms have been reported to produce thermostable xylanases.[2-6] However, none of these strains is able to produce the amounts of xylanase required for large-scale use, and in most cases they also secrete cellulases, which are undesirable in a bio-bleaching process. The properties of a protein can be changed by site-directed mutagenesis of the cloned gene, which offers interesting research opportunities.[7] Changes in enzyme characteristics, such as activity, thermostability, or pH dependency, are of great interest for the industrial application of this enzyme technology. Our studies on the xylanases from *S. lividans* indicate that xylanase A belongs to the F family according to the classification of glycanases,[8] which is based on amino acid sequence homologies found within this family. Highly conserved amino acid residues located at specific positions in xylanase A should play a role in the structure and function of enzyme, and for this reason they were targeted for site-directed mutagenesis. In this paper we describe specific mutations at codons Phe 155, Arg 156, and Asn 173 that improve the enzymatic activity and thermostability of xylanase A.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strain CJ236 *dut-1, ung-1, thi-1, rel A-1*; pCJ105 (Cm^r) harboring plasmid pUAF217 (Moreau *et al.*, manuscript submitted for publication) was used to prepare uracil-containing

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ssDNA for mutagenesis experiments. For the expression of the xylanase A gen (*xlnA*), pIAF217 was introduced into *E. coli* strain MC1061 *araD139*, *Δara-leu 7697*, *Δlac X74*, *galU-*, *galK-*, *hsr-*, *hsm-*, *strA*.

DNA manipulations and transformations

Transformation of *E. coli* and isolation of plasmid DNA were carried out as described by Sambrook *et al.*[9] Restriction and DNA-modifying enzymes were purchased from Pharmacia and used according to the manufacturer's specifications.

Site-directed mutagenesis

Mutagenesis was carried out by the method of Kunkel[10] using a commercial kit (Bio-Rad). Oligonucleotides for mutagenesis were synthesized on a Gene Assembler (Pharmacia). The primers used to mutate codons Phe 155, Arg 156, and Asn 173 are listed in *Table 1*. The double mutants E156/173D and K156/173D were generated with the N173D primer on ssDNA templates isolated from confirmed R156E and R156K mutants. Plasmid DNA was isolated from presumptive mutants and hybridized with the ^{32}P-labeled mutagenic primers in 6 × SSC at 42°C overnight, then the dot blots were washed twice for 20 min in a 3 M tetramethylammonium chloride solution at 61°C with the 21-mer oligonucleotides and at 71°C with the 27-mer oligonucleotides.[11] This base composition-independent method permits the detection of single basepair alterations generated by *in vitro* mutagenesis. Subsequently, the clones were sequenced using the dideoxy chain-termination method with ^{35}S dATP and T7 DNA polymerase according to the manufacturer's protocol (Pharmacia).

Screening of mutant transformants

The screening of mutant transformants was performed on LB plates containing ampicillin (100 μg ml^{-1}) and 0.15% oat spelt xylan (Sigma Chemical Co.) covalently linked to Remazol Brilliant Blue (Aldrich Chemical Co.).[12,13] Xylanase activity was detected by the appearance of a clear zone around the colony. *E. coli* mutant transformants were grown overnight in 25 ml of 2 × TY containing 0.5% xylose and ampicillin (100 μg ml^{-1}) at 37°C with shaking.

Xylanase assays

Xylanase activity was measured in supernatants and in periplasmic cell extracts by the dinitrosalicylic acid method.[14] One international unit (IU) was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute. The pH dependence of the xylanase activity was measured in 0.05 M citrate and Sorensen-glycine buffers for the pH ranges 4.0–8.4 and 8.0–9.0, respectively. For thermostability studies, the crude enzyme preparations were incubated at the desired temperature without substrate. Aliquots were withdrawn at given time intervals, incubated at 60°C for 10 min with 1% xylan in 0.05 M citrate buffer (pH 6.0), and the residual enzyme activities were then determined.

Table 1 List of synthetic oligonucleotides used in the mutagenesis experiments

F155Y	5'- CGCGGTGCGGTAGGCGACCTC -3'	21-mer
R156E	5'- GGCGCGCGCGGTCTCGAAGGCGACCTC -3'	27-mer
R156K	5'- GGCGCGCGCGGTCTTGAAGGCGACCTC -3'	27-mer
N173D	5'- GTTCTCGACGTCGTAGTCGTT -3'	21-mer

Underlining indicates the substituted nucleotides

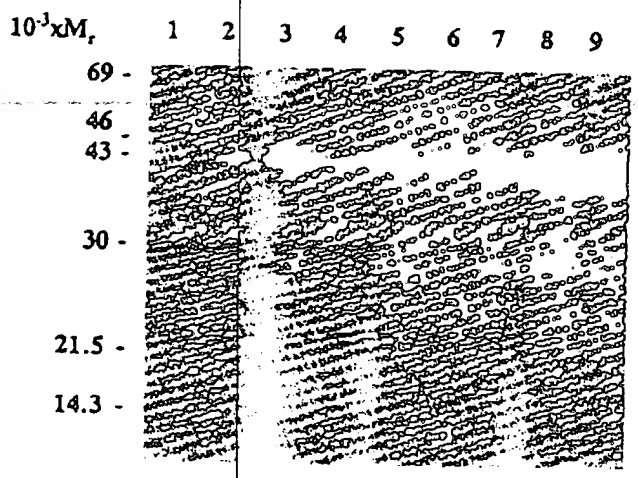

Figure 1 Protein analysis of culture supernatants of *E. coli* MC1061 transformants. Proteins were separated by SDS-PAGE (40 μg of protein was loaded onto the gel), followed by Western transfer. Immunodetection was carried out using specific anti-xylanase A antibodies coupled with ^{125}I-labeled protein A. (1) Clone with pTZ19U as control; (2) clone IAF217; (3) mutant F155Y; (4) mutant R156E; (5) mutant R156K; (6) mutant N173D; (7) mutant E156/173D; (8) mutant K156/173D; (9) native xylanase produced by *S. lividans*

Proteins and Western blot analysis

The protein content of enzyme preparations was determined according to the method of Lowry.[15] Proteins from the periplasmic space of *E. coli* cells were extracted by the chloroform shock method.[16] Proteins from culture supernatants were concentrated by precipitation with 0.2 volumes of a solution containing 50% trichloroacetic acid and 0.5% sodium deoxycholate. The mixtures were centrifuged for 10 min at 10,000 *g*, and the protein pellets were dissolved in a Tris-glycine electrophoresis buffer, pH 8.3. Proteins were analyzed by SDS-PAGE,[17] followed by Western blotting.[18] Identification of xylanase-related polypeptides was carried out with immunopurified anti-xylanase A antibodies coupled to ^{125}I-protein A (Amersham Canada Ltd.). Autoradiograms were scanned with a laser densitometer (Gelscan XL 2.1 from Pharmacia LKB) to evaluate the amount of xylanase in the samples. Purified xylanase served as a reference standard.

Results

The F family of xylanases shows DNA sequence homologies and possesses some highly conserved amino acid residues. Some of these conserved regions found in the xylanase A gene in *S. lividans* were targeted for mutations. The amino acids at positions 155, 156, and 173 were replaced either by amino acids having the same polarity but a different side chain, or by amino acids having the same side chain with a different polarity. The following mutations were carried out by site-directed mutagenesis: F155Y, R156E, R156K, N173D, E156/173D, and K156/173D. All mutants synthesized a 43-kDa protein that reacted in immunoblots with specific anti-xylanase A antibodies (*Figure 1*). The smaller immunoreactive bands are proteolytic degradation products of the recombinant xylanase. To confirm the mutation, the six mutated genes were entirely sequenced and compared to the original sequence. The only differences found were those introduced by the mutation (*Figure 2*). The sequences of E156/173D and K156/173D are not shown.

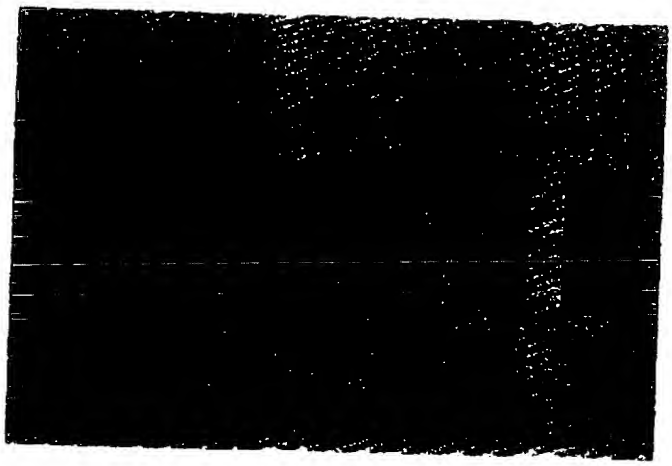

Figure 2 Sequence of *S. lividans* wild-type and mutant xylanase genes corresponding to the mutated region. Nucleotide sequences are from the noncoding strand. (1) Xylanase A wild-type sequence with the Phe codon TTC at position 155 and the Arg codon CGC at position 156; (2) mutant F155Y with the Tyr codon TAC at position 155; (3) mutant R156E with the Glu codon GAG at position 156; (4) mutant R156K with the Lys codon AAG at position 156; (5) xylanase A wild-type sequence with the Asn codon AAC at position 173; (6) mutant N173D with the Asp codon GAC at position 173. Lanes G, A, T, C of the autoradiograms correspond to the respective nucleotides. Sequence differences are indicated by arrows

Enzymatic activity of mutant xylanases

The amount of enzyme produced by each mutant was determined by immunodetection of xylanase in the culture supernatants. The results are reported in *Table 2*. The relative activity of each mutant is expressed as a percentage of that of the wild type. The specific activities were calculated from the enzyme activities obtained by the xylanase assay and autoradiogram scans by densitometry of Western blots of known enzyme concentrations. Known quantities of xylanase A were used as standards. The F155Y, R156K, R156E, and N173D xylanases showed significantly higher activities than the wild-type enzyme. Attempts to combine the favorable mutations, E156/173D and K156/173D, to generate further increases in activity were not successful. Both double mutants exhibited lower relative activities than the wild-type enzyme. Catalytic activities of wild-type and mutant xylanases were tested at different pHs. All enzymes showed nearly identical pH optima situated between pH 5.0 and 6.0 (results not shown).

Results for the optimal temperatures of mutant xylanases, determined under standard assay conditions, are shown in *Table 2*. The wild-type, R156K, E156/173D, and K156/173D xylanases shared the same optimal temperature of 65°C, those of the F155Y and N173D xylanases were 5°C lower, whereas that of the R156E xylanase was 5°C higher. The enzymatic activities of the wild-type, R156E, and R156K xylanases at 75°C were 55, 70, and 61% of their respective optimum activities. These values represent a significant increase in thermostability and might offer an advantage over the wild-type enzyme. Furthermore, to complete the thermostability studies, all mutant xylanases were tested in buffered solution at 60°C both in the presence and in the absence of substrate. The thermal inactivation curve of the enzymes without xylan is shown in *Figure 3*. The activity half-life of each mutant expressed in minutes is listed in *Table 2*. Significant differences were observed. Whereas the R156K and wild-type enzymes showed similar inactivation times, the mutant xylanases R156E and N173D both showed improved stability, and F155Y was considerably less stable. Of the two combination mutants, the E156/173D xylanase was twice as stable as the wild type. As it is known that the substrate often protects the enzyme from thermal inactivation to some extent, enzyme thermostability was also tested in the presence of xylan. The enzymes were thus also incubated in the presence of 3% birchwood xylan at 60 and 70°C, and the liberation of reducing sugar was assayed to determine enzymatic activity. The results are shown in *Figure 4*. At 60°C, the activity curve of all xylanases was linear for 5 h, indicating that the substrate exerted a general protective effect on the enzymes, independent of the type of mutation. These values were considerably higher than those observed for half-lives measured in the absence of substrate at the same temperature (*Table 2*). The same experiment carried out at 70°C revealed that the protection due to the substrate was shortened to 30 min for the wild-type, N173D, F155Y, E156/173D, and K156/173D

Table 2 Properties of wild-type and mutant xylanases from *Escherichia coli* supernatant

Mutant	Relative activity (%)[a]	Optimum temperature[b]	pH optimum	Half-life (min)[c]
IAF217 (wt)	100	65	6.0	110
F155Y	128	60	6.0	45
R156E	110	70	6.0	116
R156K	150	65	6.0	110
N173D	125	60	6.0	150
E156/173D	78	65	6.0	220
K156/173D	53	65	6.0	110

[a]Activity was determined according to the mean of three assays with 1% xylan in 0.05 M citrate buffer pH 6.0 at 60°C for 10 min with 400 μg of protein from the culture supernatants of the clones. The amount of xylanase in the supernatants was evaluated by laser scan densitometry of Western blot autoradiograms, and the specific activity was then determined. The relative activity was the ratio of the specific activity of each mutant to specific activity of the wild-type multiplied by 100

[b]Optimum temperatures were determined using 1% xylan in 0.05 M citrate buffer, pH 6.0, for 10 min at various temperatures

[c]Thermostabilities were measured by incubating the enzyme preparations without substrate at 60°C. At given times, aliquots were withdrawn and the remaining activity was measured at 60°C with 1% xylan in 0.05 M citrate buffer, pH 6.0, for 10 min

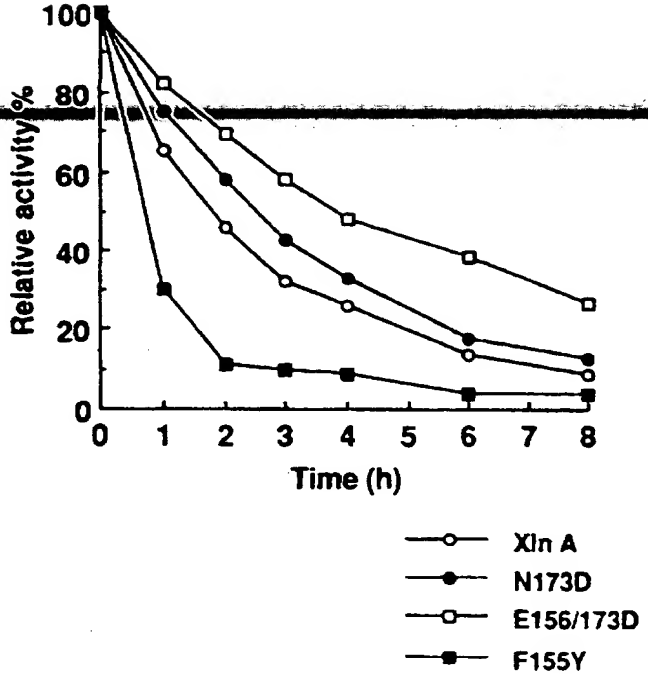

Figure 3 Thermostability of the mutant xylanases in absence of substrate at 60°C. Culture supernatants of clones were preincubated at 60°C for various lengths of time and remaining activities were determined at the same temperature in 0.05 M citrate buffer, pH 6.0, for 10 min with 1% birchwood xylan. The values are expressed as percentages of the initial activity. (○) Xln A; (●) Xln N173D; (□) Xln E 156/173D; (■) Xln F155Y

xylanases. However, the R156K and R156E xylanases were protected for 60 and 90 min, respectively (*Figure 4*).

Discussion

Streptomyces lividans produces three xylanases which are well characterized.[14,19,20] The homologous cloning of the xylanase A gene in *S. lividans* yielded clones which secrete 2 $g\,l^{-1}$ or more of xylanase.[21] For this reason, they are considered good candidates for large-scale industrial production of the enzyme. The xylanase A of *S. lividans* is very active and is reasonably thermostable. Still, we considered it of interest to attempt to improve some properties of the enzyme by site-directed mutagenesis of the xylanase A gene.

Thermostability and improved catalytic activity were targeted in these studies. The improvement in stability obtained by replacing the arginine situated at position 156 of xylanase A by a glutamic acid residue (R156E) resulted in a modified enzyme with a temperature optimum that was 5°C higher than that of the wild type (*Table 2*). This result could partly be expected from the DNA sequence homologies of the F family. In fact, the same substitution occurs naturally in a xylanase produced by *Bacillus* sp. C-125 and in a xylanase produced by *Caldocellum saccharolyticum*.[8] Both xylanases have an optimum temperature of 70°C, which is the same as for R156E.[22,23] However, in *Thermoascus aurantiacus*, where an alanine resides in this position, the optimal temperature is also at 70°C.[24] On the other hand, the xylanases produced by *Clostridium thermocellum* (*xynZ*), *Cryptococcus albidus*, and the exoglucanase from *Cellulomonas fimi* all have an arginine residue at position 156 and have an optimum temperature of 65°C or less.[25-27] Therefore, we can conclude that the replacement of arginine at position 156 by either glutamic acid or alanine influences the thermostability of the xylanases. There is apparently no direct relationship between optimum temperature and thermostability. At 60°C, in the absence of substrate, the R156E,

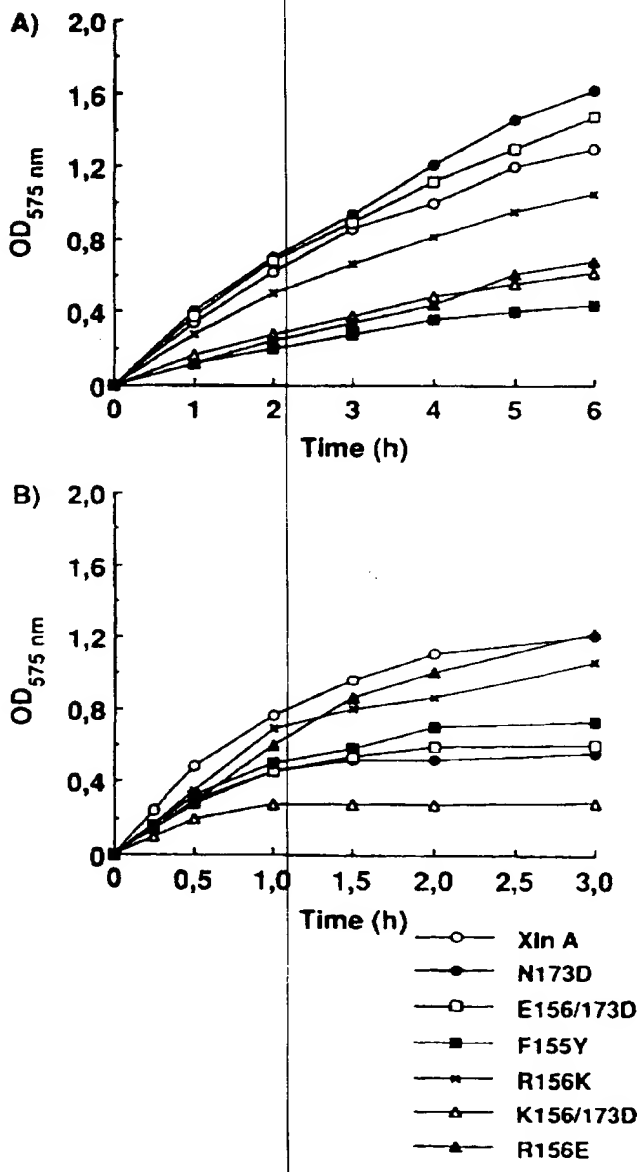

Figure 4 Thermostability of mutant xylanases in the presence of 3% birchwood xylan. Cultur supernatants of clones were incubated at 60°C (A) or at 70°C (B) in presence of 3% birchwood xylan in a citrate buffer pH 6.0, for various lengths of time. Aliquots were taken and the amount of sugar liberated was measured by a reducing sugar test. (○) XlnA; (●) Xln N173D; (□) Xln E156/173D; (■) Xln F155Y; (X) Kln R156K; (△) Kln K156/173D; (▲) Xln R156E

N173D, and the double mutant R156/173D xylanases were, in increasing order more stable than the wild-type enzyme. The fact that the R156E xylanase had a higher optimal temperature did not appear to significantly influence its stability. Similar results were reported in the case of two thermostable cellulases from *Trichoderma reesei* TD beta-6 and *Thielavia terrestris* NRRL 8126.[28] Combining the two most favorable mutations, R156E and N173D, led to more than a simple additive effect on thermostability. The resulting enzyme was twice as stable at 60°C as the wild type, with a half-life of 220 min, and considerably more stable than that of the single mutants. The codons Phe 155 and Asn 173 were not combined, since the F155Y xylanase was more sensitive to thermal inactivation (*Table 2*) and to proteolytic degradation (data not shown). This observation was in good agreement with data suggesting a correlation between protein thermostability and resistance to proteolysis.[29] Chou-Fasman and Robson-Garnier prediction programs were used to determine the mean hydrophobicity of regions containing the mutation. The substitution Asn 173 for Asp significantly increased the mean hydrophobicity, while the replacements at codons Phe 155 and Arg 156 had little or no effect. An increase in hydrophobicity in a region of a protein should reduce the number of water molecules having access to this locus or create a more compact protein as result of new secondary structure interactions. This phenomenon could eventually increase protein thermostability, as shown for the N173D xylanase.

In general, the presence of substrate stabilizes the enzymes. Xylanase activity was protected for 5 h at 60°C in the presence of 3% xylan, independently of the introduced mutations. However, at 70°C, the R156E xylanase was the most stable enzyme (*Figure 4B*). The question arises: "Which of these mutant xylanases is the most suitable for a biobleaching process for paper pulp?" Paper pulp contains considerable amounts of xylan which should be sufficient to protect enzymes against thermal inactivation during treatment. Data on enzyme thermostability in the presence of pulp are not yet available, but results presented in this paper suggest the N173D or E156/173D mutant enzymes, which are more stable than the wild-type enzyme in the absence of substrate, could be used.

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References

1 Paice, M.G. and Jurasek, L. Removing hemicellulose from pulps by specific enzymic hydrolysis. *J. Wood Chem. Technol.* 1984, 4, 187–198
2 Grüninger, H. and Fiechter, A.A. A novel, highly thermostable D-xylanase. *Enzyme Microb. Technol.* 1986, 8, 309–314
3 Tan, L.U.L., Mayers, P. and Saddler, J.N. Purification and characterization of a thermostable xylanase from thermophilic fungus *Thermoascus auranticus*. *Can. J. Microbiol.* 1987, 33, 689–692
4 Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N. Multiplicity of β-1,4 xylanase in microorganisms: Functions and applications. *Microbiol. Rev.* 1988, 52, 305–317
5 Simpson, H. D., Haufler, U.R. and Daniel, R.M. An extremely thermostable xylanase from thermophilic eubacterium *Termotoga*. *Biochem. J.* 1991, 277, 413–417
6 Zamost, B.L., Nielsen, H.K. and Starnes, R.L. Thermostable enzymes for industrial applications. *J. Indust. Microbiol.* 1991, 8, 71–82
7 Estell, D. A., Graycar, T.P. and Well, J.A. Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. *J. Biol. Chem.* 1985, 260, 6518–6521
8 Shareck, F., Roy, C., Yaguchi, M., Morosoli, R. and Kluepfel, D. Sequences of three genes specifying xylanases in *Streptomyces lividans*. *Gene* 1991, 107, 75–82
9 Sambrook, J., Fritsch, E. and Maniatis, T. In: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989
10 Kunkel, T.A. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* 1985, 74, 488–492
11 Wood, W.I., Gitschier, J., Lasky, L.A. and Lawn, R.M. Base-composition-independent hybridization in tetramethylammonium chloride: A method for oligonucleotide screening of highly complex gene libraries. *Proc. Natl. Acad. Sci. USA* 1985, 82, 1585–1588
12 Biely, P., Mislovicova, D. and Toman R. Sensitive detection of endo-1,4-β-xylanases in gels. *Anal. Biochem.* 1985, 144, 142–146
13 Kluepfel, D. Screening of prokaryotes for cellulose- and hemicellulose-degrading enzymes. *Methods Enzymol.* 1988, 160, 180–186
14 Kluepfel, D., Daigneault, N., Morosoli, R. and Shareck, F. Purification and characterization of new xylanase (xylanase C) produced by *Streptomyces lividans* 66. *Appl. Microbiol. Biotechnol.* 1992, 36, 626–631
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951, 193, 265–275
16 Ames, G.F.L., Prody, C. and Kustu, S. Simple, rapid and quantitative release of periplasmic proteins by chloroform. *J. Bacteriol.* 1984, 160, 1181–1183
17 Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970, 227, 680–685
18 Towbin, H., Staehelin, T. and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 1979, 76, 4350–4354
19 Morosoli, R., Bertrand, J.L., Mondou, F., Shareck, F. and Kluepfel, D. Purification and properties of a xylanase from *Streptomyces lividans*. *Biochem. J.* 1986, 239, 587–592
20 Kluepfel, D., Vats-Mehta, S., Aumont, F., Shareck, F. and Morosoli, R. Purification and characterization of a new xylanase (xylanase B) produced by *Streptomyces lividans* 66. *Biochem. J.* 1990, 267, 45–50
21 Bertrand, J.-L., Morosoli, R., Shareck, F. and Kluepfel, D. Expression of the xylanase gene of *Streptomyces lividans* and production of the enzyme on natural substrates. *Biotechnol. Bioeng.* 1989, 33, 791–794
22 Honda, H., Kudo, T., Ikura, Y. and Horikoshi, K. Two types of xylanases of alkalophilic *Bacillus* sp No. C-125. *Can. J. Microbiol.* 1985, 31, 538–542
23 Lüthi, E., Love, D.R., McAnulty, J., Wallace, C., Caughey, P.A., Saul, D. and Bergquist, P.L. Cloning, sequence analysis and expression of genes encoding xylan-degrading enzymes from the thermophile *Caldocellum saccharolyticum*. *Appl. Environ. Microbiol.* 1990, 56, 1017–1024
24 Srinivasa, B.R., Swaminathan, K.R., Ganapathy, C., Roy, R.P., Murthy, S.K. and Vithayathil, P.J. The primary structure of xylanase from *Thermoascus aurantiacus*. *Protein Seq. Data Anal.* 1991, 4, 15–20
25 Grépinet, O., Chebrou, M.C. and Beguin, P. Purification of *Clostridium thermocellum* xylanase Z expressed in *Escherichia coli* and identification of the corresponding product in the culture medium of *C. thermocellum*. *J. Bacteriol.* 1988, 170, 4576–4581
26 Morosoli, R., Roy, C. and Yaguchi, M. Isolation and partial primary sequence of a xylanase from the yeast *Cryptococcus albidus*. *Biochim. Biophys. Acta* 1986, 870, 473–478
27 O'Neil, G.P., Goh, S.H., Warren, R.A.J., Kilburn, D.G. and Miller, R.C., Jr. Structure of the gene encoding the exoglucanase of *Cellulomonas fimi*. *Gene* 1986, 44, 325–330
28 Durand, H., Soucaille, P. and Tiraby, G. Comparative study of cellulases and hemicellulases from four fungi: Mesophiles *Trichoderma reesei* and *Penicillium* sp. and thermophiles *Thielavia terrestris* and *Sporotrichum cellulophilum*. *Enzyme Microb. Technol.* 1984, 6, 175–180
29 Daniel, R.M., Cowan, D.A., Morgan, H.W. and Curran, M.P. A correlation between protein thermostability and resistance to proteolysis. *Biochem. J.* 1982, 207, 641–644